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Partial Purification of the Molybdenum-reducing Enzyme from *Bacillus pumilus* strain Lbna

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ABSTRACT

Molybdenum is an emerging pollutant. Bioremediation of this heavy metal is possible by the mediation of Mo-reducing bacteria. These bacteria contain the Mo-reducing enzymes that can conver toxic soluble molybdenum into molybdenum blue; a less soluble and less toxic form of the metal. To date only the enzyme has been purified from only one bacterium. The aim of this study is to purify the Mo-reducing enzyme from a previously isolated Mo-reducing bacterium *Bacillus pumilus* strain Lbna using ammonium sulphate fractionation followed by ion exchange and then gel filtration. Two clear bands were obtained after the gel filtration step with molecular weights of 70 and 100 kDa. This indicates that further additional purification methods need to be used to get a purified fraction. Hence, additional steps of chromatography such as hydroxyapatite or chromatofocusing techniques can be applied in the future.

INTRODUCTION

Bacterial based remediation of environmental toxicants utilizes mechanisms such as bioreduction, bioprecipitation, bioaccumulation or sequestration, efflux pumping and biosorption. This technology is a promising innovative technology that has attracted great interest in recent years [1,2]. Metal ions that can be bioremediated include metals such as molybdenum, chromium, copper and mercury [3–6]. Of these metals, molybdenum is an emerging pollutant, and microbial molybdate (Mo^{6+}) reduction to the less toxic molybdenum blue has been reported by Capaldi and Proskauer [7] over a century ago in *E. coli*.

The mechanism of enzymatic molybdate reduction to molybdenum blue was earlier proposed to involve enzyme catalyzed redox changes in the oxidation state of molybdenum (Mo^{6+} to Mo^{5+}) before the addition of phosphate which leads to the formation of molybdenum blue by several researchers [8,9]. However, the new evidence demonstrated the possibility of the involvement of the intermediate phosphomolybdate [10].

The term molybdenum-reducing activity was used rather than molybdenum reductase [8] because the enzyme responsible for molybdate reduction has not been previously characterized. [10] partially purify molybdenum-reducing enzyme from *E. cloacae* Strain 48 by ammonium sulfate (40-50%) fractionation and ion-exchange on DE-cellulose, and gel filtration on Sephacryl S-200 with a final 6.5-fold enzyme purification and about 97% recovery of the initial value was achieved. As of now, the molybdenum-reducing enzyme has been purified to homogeneity, but the yield was poor [11]. The apparent molecular weight of the Mo-reducing enzyme was estimated from gel filtration to be 105 kDa, and the enzyme is monomeric. Similarly, a marked decrease in enzyme yield resulting from ion-exchange chromatography as observed in several previous works [11]. The poor yield delays the enzyme sequencing and hence the enzyme nomenclature.

It is important that newer sources of the molybdenum-reducing are obtained and characterized so that comparisons can be made, and the best enzyme can then be used in further improvement studies such as in genetic engineering for efficient bioremediation. This is the aim of this study; to partially purify the enzyme from a previously isolated Mo-reducing bacterium [12].

MATERIALS AND METHODS

Low phosphate media (LPM)

LPM (pH 7.0) was prepared in 1 litre of deionised water using the ingredients as followed: 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 5 g of NaCl, 2.42 g of $Na_2MoO_4.2H_2O$, 0.73 g of $Na_2HPO_4$and 10 g of glucose. The medium was autoclaved at 121°C at 115 kPa for 15 minutes, and glucose was autoclaved separately [12].

High phosphate media (LPM)

The chemicals needed for HPM were the same as LPM, but HPM had the significantly higher amount of phosphate compared to LPM. The chemicals needed to make HPM in 1 litre of deionised water were as followed: 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 5 g of NaCl, 5 g of $Na_2MoO_4.2H_2O$, 14.6 g of $Na_2HPO_4$and 10 g of glucose. The pH of the media was fixed to 7.5. It was autoclaved at 121°C at 115 kPa for 15 minutes before usage. It is important to note that glucose was autoclaved separately, and only added to the autoclaved medium before inoculation [12].

Preparation of Crude Enzyme

Growth of *Bacillus pumilus* strain Lbna [12] was carried out at high phosphate molybdate media since growth at the optimum phosphate concentration resulted in the production of molybdenum blue that complicates enzyme preparation. Although the high phosphate inhibits molybdate reduction to molybdenum blue, the cells contain active enzymes [11]. The following experiment was carried out at 4ºC unless stated otherwise. Cells were initially harvested by centrifugation at 10 000 g for 10 minutes.

Then, the cells were washed thrice with distilled water. The cells were then resuspended and recentrifuged at 10 000 g for 10 minutes. The pellet was reconstituted with 10 ml of 50 mM Tris buffer pH 7.0. The cells were then sonicated for 1 minute on an ice bath with 4 minutes cooling until a total sonication time of at least 20 minutes was achieved. The sonicated fraction was centrifuged at 10 000 g for 20 minutes, and the supernatant consisting of the crude enzyme fraction was taken.

Mono Q Ion-exchange

Ion-exchange chromatography was chosen as it is low cost, able to accommodate high amounts of protein, able to cope with large sample volume and the ability to concentrate protein from dilute solution prior to elution process (Scopes, 1988). Due to these properties, ion-exchange is the most popular fractionation method employed after ammonium sulphate fractionation (Scopes, 1988). Preliminary studies show that the Mo-reducing enzyme does not bind to cation-exchanger such as carboxymethyl-cellulose or CM-cellulose and thus an anion-exchanger; strong quarternary ammonium exchanger MonoQ was used instead. The experiments were carried out as follows:

In the following experiments involving Mo-reducing enzyme purification proteins eluted were monitored using a UV flow-through monitor set at 280 nm (Bio-Rad) and collected using a fraction collector (Bio-Rad). Peak profile was automatically recorded on a chart recorder (Gilson).

Column Chromatography Preparation

Mono Q (GE healthcare) with a 1 ml bed volume was washed with 200 ml of buffer A until the eluant has a pH of pH 7.5. The crude fraction (2 ml) was loaded onto the column and washed with 30 ml of buffer A. Two millilitres fractions were collected and assayed for Mo-reducing enzyme activity in the unbound fraction. A linear elution gradient of sodium chloride of between 0 to 1000 mM NaCl (50 ml) in buffer A was set up in a gradient mixer. Two millilitres of fractions were collected and assayed for the Mo-reducing enzyme in the eluted fractions.

Gel Filtration on Zorbax GF-250

Gel filtration chromatography was used for the last step of enzyme purification due to its excellent fractionation power based on molecular weight but very limited sample capacity. Gel filtration separates protein based on the size of the protein. The molecular size of the sample molecule is related to its molecular weight. The Zorbax^R Bioseries GF-250 from Agilent is a pre-packed gel filtration column with 4 µm particle diameter spherical silica and recommended for separating proteins over the 4,000 to 400,000 Da range. Preliminary studies showed that blue dextran (average mwt of 2 million Dalton) elutes out at 8 ml and this is the void volume (Vo) of the column.

The manufacturer claimed that the total volume (Vt) is 13 ml. and The bed dimensions are 2.50 cm x 0.94 cm and the bed volume are approximately 23.5 ml with a pore diameter of 150 Å. The range of workable pH buffer for this matrix is between pH 3.5-8.5. The Zorbax^R Bioseries GF-250 column was equilibrated with 120 ml of 0.02 M phosphate buffer (pH 7.5) containing 0.2 M NaCl. Salt is added as recommended by the manufacturer to prevent nonspecific hydrophobic binding of the protein to this column. The sample was loaded with 150 µl of concentrated Mono-Q^{TM} eluent containing 0.5 mg protein. Preliminary studies using Tris buffer inexplicably gave lower yield; thus the use of phosphate buffer.

The enzyme was eluted from the column with the same buffer at a flow rate of 0.5 ml/min. The eluent was collected using a fraction collector at 1 ml per tube and assay for enzyme activity and protein content. The protein elution profile was monitored using HPLC UV detector at 280 nm. Fraction containing enzyme activity was collected and concentrated.

SDS-polyacrylamide gel electrophoresis

The purified Molybdenum reductase subunit was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) under denaturing nonreducing conditions using aPROTEAN® 3 CELL apparatus (BioRad). Stock sample buffer was prepared in small aliquots of 1.0 ml in Eppendorf™ tubes each containing 0.06 M Tris-HCl pH 6.8, 10% glycerol and 0.025% Bromophenol blue (Bio-Rad) and stored at –20ºC. Samples were heated for 5 minutes in sample buffer.

Once the protein bands have been separated on the gel, they were visualized using Coomassie Brilliant Blue R-250 (Bio-Rad). Broad range protein standard marker (BioLabs) was used to determine the molecular weight of the protein. The broad range SDS-PAGE protein markers used in order of decreasing molecular mass were Myosin (212, 000), MBP-β-galactosidase (158, 194), β-Galactosidase (116, 351), Phosphorylase b (97, 184), Serum albumin (66, 409), Glutamic

dehydrogenase (55, 561), MBP2 (42, 710), Thioredoxin reductase (34, 622) and Triosephosphate isomerase (26, 972).

Gel Staining

Coomassie Brilliant Blue R-250 staining solution was prepared by dissolving one gram of Coomassie Brilliant Blue R-250 in 200 mL solution of 40% methanol (v/v) and 10% acetic acid (v/v). The solution was filtered through a Whatman™ no. 3 filter paper. Part of the SDS-PAGE containing the markers was soaked in 150 mL of the solution overnight and destained with a large excess of destaining solution containing 40% methanol (v/v) and 10% acetic acid (v/v) (Laemmli, 1970) until a series of marker bands appeared from the gel background. Coomassie blue R-250 staining solution was used when proteins were relatively abundant and thus sample from the supernatant after ultracentrifugation and affinity chromatography was stained with Coomassie blue R-250 staining solution [13].

Protein determination

Protein concentration was determined using the method of Bradford [14]. The Coomassie blue dye binds to basic and aromatic amino acids resulting in a shift of the absorbance maximum from 465 nm to 595 nm. The amount of protein present is standardized by using bovine serum albumin (BSA).

The number of proteins in samples was measured by adding 20 µl of suitably diluted sample into a microtiter plate well containing 200 µl of Bradford reagent (diluted 10×). The mixture, at a final volume of 220 µl was incubated for 5 minutes before the absorbance was read on the Omnitek microtiter plate reader at the wavelength of 600 nm. All Samples were run in triplicates.

RESULTS AND DISCUSSION

Partial Purification of Mo-reducing Enzyme

The partial purification of Mo-reducing enzyme was carried out using the concentrated supernatant. This enzyme was purified using ion exchange chromatography (Mono Q) and gel filtration chromatography (Zorbax GFC-250). First, the concentrated supernatant (5 mg/ml) prepared from the enzyme crude extract was eluted into the Mono Q with a concentrated gradient of NaCl 1mM.

The elution profile of ion exchanger chromatography shows a peak contain enzyme activity and elutes at tubes number between 9 to 13 (**Fig**. **1**). These fractions were collected and dialysed in 1L of tris Buffer ph 7.5 for 1 hour repeated 3 times. These fractions were concentrated to 300µl on an Amicon concentrator and was injected into a gel filtration column. The elution profile from gel filtration column shown in (**Fig**. **2**) showed four peaks between tubes 3 to 30. Tube 20 to 22 had the highest specific enzyme activity. The detail step protein content and activity in each purification step are summarized in Mo-reducing purification table (**Table 1**).

Table 1. Summary of Mo-reducing enzyme purification.

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Percentage yield (%)	Fold purification
Crude	0.0898	294	3273.9	100	1
Ion-Exchange Chromatography (Mono-Q)	0.01579	91.796	5813.6	31.22	1.776
Gel Filtration Chromatography (Zorbax GFC-250	0.0314	64.548	2055.7	21.955	0.627

The molybdenum enzyme is very sensitive towards temperature that enzyme yield was low after each chromatography steps. The purification fold also decreases when gel filtration step was employed because the enzyme was denatured, but protein content was high.

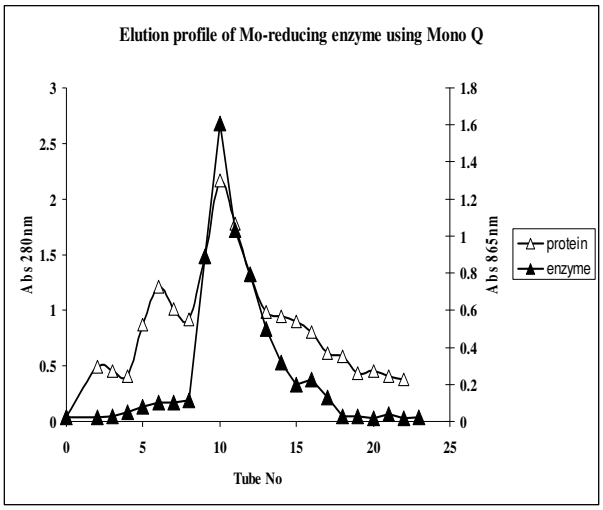

Fig. 1. Elution profile of Mo-reducing enzyme from *Bacillus pumilus* strain Lbna using Ion exchange chromatography (Mono Q).

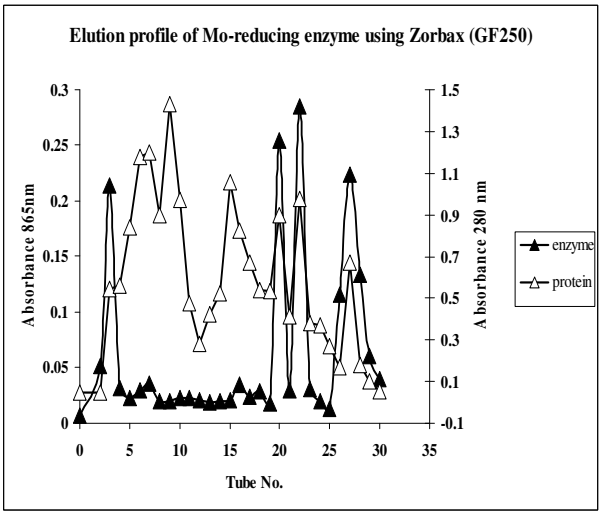

Fig. 2. Elution profile of Mo-reducing enzyme from *Bacillus pumilus* strain Lbna on Zorbax GFC-250.

SDS Polyacrylamide Gel Electrophoresis

Electrophoresis of protein for each purification step was demonstrated by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE). SDS-PAGE is used after each fractionation step to monitor the increase in purity of Mo-reducing enzyme, observed by the lessening of bands. **Fig. 3** shows the SDS-PAGE results for samples of Mo-reducing enzymes obtained after several purification steps. The number of protein bands decreased significantly throughout the purification steps.

The gel shows that the number of protein bands after gel filtration are the least. Two clear bands were determined after the gel filtration sample with the molecular weights of 70 and 100 kDa. In a previous study of molybdenum reducing enzyme from *E. cloacae* strain 48, the enzyme was purified using ammonium sulphate filtration, DEAE-cellulose ion-exchange

chromatography and Sephacryl S-200 gel filtration, which also shows three bands with higher values of molecular weight, which are 80, 90 and 100 kDa [15]. The presence of the 100 kDa band is worth noting as the purified Mo-reducing enzyme from *Serratia* sp. Strain Dr.Y5 showed a similar 105 kDa band [11].

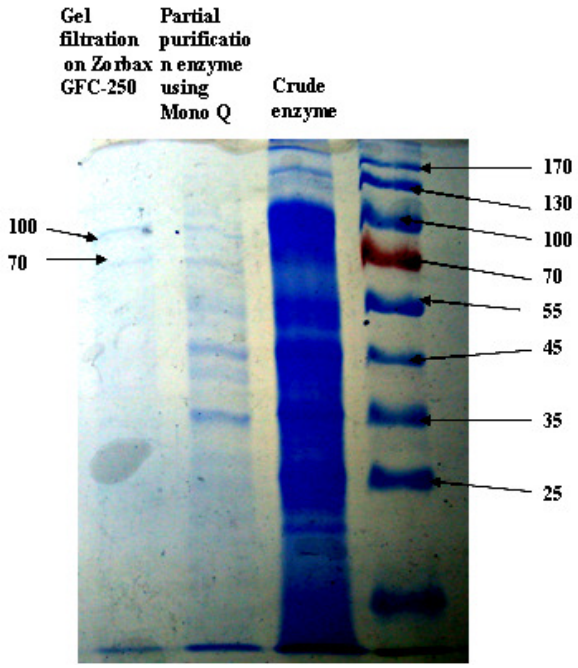

Fig. 3. Electrophoresis analysis of the crude and partially purified Mo-reducing enzyme from *Bacillus pumilus* strain Lbna in a 7.5 % polyacrylamide gel. Protein bands were stained using Coomassie Brilliant Blue R-250 dye. Lane marker; molecular weight markers; Lane A, crude Mo-reducing enzyme; Lane B, partially purified Mo-reducing enzyme.

CONCLUSION

The Mo-reducing enzyme from *Bacillus pumilus* strain Lbna has been successfully partially purified, albeit not to homogeneity. An interesting observation is the presence of a 100 kDa protein band, which is closely similar to the Mo-reducing enzyme previously purified from another Mo-reducing bacterium. This indicates that a further additional purification method can be used in the hope to get a purified fraction. Generally, gel filtration is the last step of purification due to the limited amount of protein that it can handle. Hence, an additional step of chromatography such as hydroxyapatite or chromatofocusing technique can be applied in the future.

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